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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

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INVENTOR(S)		
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☒ *Additional inventors are being named on the 1 separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

METHOD FOR IMAGING AND DIFFERENTIAL ANALYSIS OF CELLS

CORRESPONDENCE ADDRESS

Direct all correspondence to:

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 24 ☐ CD(s), Number

☒ Drawing(s) *Number of Sheets* 5 ☒ Other (specify) Fee Transmittal (+ copy)

☒ Application Data Sheet. See 37 CFR 1.76

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☒ Applicant claims small entity status. See 37 CFR 1.27.

☒ A check or money order is enclosed to cover the filing fees. Filing Fee Amount: $80

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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Respectfully submitted,			
SIGNATURE		DATE 3/16/04	March 16, 2004
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PROVISIONAL APPLICATION COVER SHEET
Additional Page

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Docket No.	120122.405P1

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☒ Applicant claims small entity status. See 37 CFR 1.27.

TOTAL AMOUNT OF PAYMENT ($) 80

Complete If Known	
Application Number	
Filing Date	
First Named Inventor	Thaddeus C. George
Examiner Name	
Art Unit	
Attorney Docket No.	120122.405P1

METHOD OF PAYMENT

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee($)	Small Entity Fee Code	Small Entity Fee($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80

SUBTOTAL (1) ($) 80

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

			Extra Claims		Fee from below	=	Fee Paid
Total Claims		-** =		*		=	
Independent Claims		-** =		*		=	
Multiple Dependent						=	

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** *Reissue independent claims over original patent*
1205	18	2205	9	** *Reissue claims in excess of 20 and over original patent*

SUBTOTAL (2) ($)

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FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet.	
1053	130	1053	130	Non-English specification	
1812	2520	1812	2520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1840*	1805	1840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1480	2254	740	Extension for reply within fourth month	
1255	2010	2255	1005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1510	1451	1510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive – unavoidable	
1453	1330	2453	665	Petition to revive – unintentional	
1501	1330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR § 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR § 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	
Other fee (specify) ____					

*Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($)

SUBMITTED BY

Name (Print/Type)	Karl R. Hermanns	Registration No. Attorney/Agent)	33,507	Customer Number 00500
Signature		Date	March 16, 2004	

465226

METHOD FOR IMAGING AND DIFFERENTIAL ANALYSIS OF CELLS

BACKGROUND OF THE INVENTION

Field of the Invention

The present disclosure relates generally to imaging small moving objects or particles to analyze and distinguish such objects, and more specifically, to a system and method for determining and analyzing photometric and morphogenic features of small objects, such as cells to, for example, identify different cell states.

Description of the Related Art

Apoptosis is a complex, tightly regulated process by which a cell orchestrates its own destruction in response to specific internal or external triggers (Jacobson *et al.*, *Cell 88*:347, 1997; Rathmell and Thompson, *Cell 109 (Supp)*:S97, 2002) and proceeds in a manner that is designed to prevent damage to surrounding cells and tissues. Apoptotic cells typically appear shrunken, with condensed chromatin and fragmented nuclei. Although plasma membrane integrity is initially preserved, in later stages the plasma membrane becomes compromised and the cells shed apoptotic bodies consisting of organelles, cytoplasm and/or nuclear fragments. Apoptotic cells are rapidly phagocytosed and eliminated *in vivo*, thus preventing the induction of inflammatory responses and this process is critical to the maintenance of tissue and immune cell development and homeostasis (Jacobson *et al.*; Rathmell and Thompson; Vaux and Korsmeyer, *Cell 96*:245, 1999). Inappropriately low apoptotic rates can result in cancer or autoimmune disease, while high rates can result in neurodegenerative disease or immunodeficiency (Ashkenazi and Dixit, *Science 281*:1305, 1998; Thompson, *Science 267*:1456, 1995; Fadeel *et al.*, *Leukemia 14*:1514, 2000). In contrast, necrotic cell death is a largely unregulated process in which the cells generally have intact nuclei with limited chromatin condensation. Cells undergoing necrosis do not induce an early phagocytic response. Instead, the cells swell and rupture, and the release of cellular contents can result in significant local tissue damage and inflammation (Jacobson *et al.*).

Research aimed at cell death regulation has produced a number of methods to identify and quantify apoptotic cells, and to distinguish between cells undergoing apoptosis versus necrosis. Among these, flow cytometry has become a commonly used tool in the identification and quantification of apoptosis. Changes in cell size, shape, and granularity associated with apoptosis can be inferred from scattered laser light (Ormerod *et al.*, *J. Immunol. Methods 153*:57, 1992). Early intracellular events, such as the loss of the mitochondrial inner membrane potential or activation and cleavage of caspases, can also be detected using electro-potential sensitive dyes (Castedo *et al.*, *J. Immunol. Methods 265*:39, 2002; Green and Kroemer, *Trends Cell. Biol. 8*:267, 1998; Green and Reed, *Science 281*:1309, 1998; Kroemer and Reed, *Nat. Med. 6*:513, 2000; Lizard *et al.*, *Cytometry 21*:275, 1995) or fluorogenic substrates (Komoriya *et al.*, *J. Exp. Med. 191*:1819, 2000; Smolewski *et al.*, *J. Immunol. Methods 265*:111, 2002; Lecoeur *et al.*, *J. Immunol. Methods 265*:81, 2002). Another early apoptotic event results in exposure of phosphatidylserine on the outer surface of the plasma membrane, which can be detected by fluorochrome-labeled annexin V (van Engeland *et al.*, *Cytometry 31*:1, 1998; Vermes *et al.*, *J. Immunol. Methods 184*:39, 1995; Koopman *et al.*, *Blood 84*:1415, 1994; Verhoven *et al.*, *J. Exp. Med. 182*:1597, 1995). Apoptotic cells eventually lose the ability to exclude cationic nucleotide-binding dyes and nuclear DNA stains with dyes, such as propidium iodide and 7-aminoactinomycin D (7-AAD) (Lecoeur *et al.*, 2002; Gaforio *et al.*, *Cytometry 49*:8, 2002; Ormerod *et al.*, *Cytometry 14*:595, 1993; Schmid *et al.*, *J. Immunol. Methods 170*:145, 1994; Philpott *et al.*, *Blood 87*:2244, 1996). Other techniques that can be used to identity apoptosis include biochemical identification of the activated proteases (*e.g.*, caspases, PARP), release of mitochondrial cytochrome c, quantification of cellular DNA content, and progressive endonucleolytic cleavage of nuclear DNA (Alnemri *et al.*, *Cell 87*:171, 1996; Kohler *et al.*, *J. Immunol. Methods 265*:97, 2002; Gong *et al.*, *Anal. Biochem. 218*:314, 1994; Gorczyca *et al.*, *Leukemia 7*:659, 1993; Gorczyca *et al.*, *Cancer Res. 53*:1945, 1993).

As noted above, conventional flow cytometric methods do not provide direct morphologic evidence of cell death. Indeed, these techniques usually target molecular changes that are associated with apoptosis, but such changes are not always specific to apoptosis and

may also be present in cells undergoing necrotic death (Lecoeur *et al.*, 2002; Lecoeur *et al.*, *Cytometry 44*:65, 2001; Kerr *et al.*, *Br. J. Cancer 26*:239, 1972). For example, necrotic cells, like advanced (late-stage) apoptotic cells, stain with both annexin V and 7-AAD (Lecoeur *et al.*, 2002; Lecoeur *et al.*, 2001). Thus, visualization of the characteristic morphologic changes associated with apoptosis is still considered to be absolutely necessary for its identification (Jacobson *et al.*; Darzynkiewicz *et al. Cytometry 27*:1, 1997). Standard microscopic techniques allow visualization of specific molecular and biochemical changes associated with apoptosis and also morphologic changes that distinguish apoptosis from necrosis. However, these standard techniques also require subjective analysis and time-consuming image viewing, which only allows for processing of relatively limited numbers of cells and, therefore, makes it difficult to attain statistically valid comparisons (Tarnok and Gerstner, *Cytometry 50*:133, 2002).

Thus, the need exists for techniques that can provide the statistical power offered by flow cytometry coupled with the objective assessment capabilities associated with microscopic analysis. For example, interest in the dynamic nature of the living cell and efforts to model cell processes (variously termed "cytomics" or "systems biology") are powerful drivers for new techniques to acquire ever more comprehensive data from cells and cell populations. The present invention meets such needs, and further provides other related advantages.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic representation of the ImageStream 100™ multispectral imaging cytometer.

Figures 2A – 2C show an analysis of cell death using standard flow cytometry and immunofluorescence microscopy. Untreated Jurkat cells (A), Jurkat cells treated with 1 μM CPT for 18 hrs (B), and Jurkat cells treated with 0.3% hydrogen peroxide for 1 hour (C) were stained with Alexa Fluor 488 conjugated annexin V and 7-AAD. Cells were analyzed either by conventional flow cytometry (using a BD FACSort™) or visualized on slides using a Nikon Eclipse E600 fluorescence microscope equipped with bandpass filters appropriate for

Alexa Fluor 488 (535/40 nm) and 7-AAD (630/60 nm) fluorescence. The 2-color dot-plots of annexin V vs. 7-AAD, and the brightfield, combined fluorescence and darkfield microscopic images are shown.

Figures 3A – 3E show flow cytometric imaging of untreated, CPT-treated and peroxide-treated Jurkat cells that were stained with Alexa Fluor 488 conjugated annexin V and 7-AAD. Peroxide-treated cells were also separately stained with HLA class I-PE. After staining, equal cell numbers of the three populations of cells were mixed and analyzed by (A) conventional flow cytometry using a FACSort™; and (B) multispectral imaging of cells in flow using an ImageStream 100™ cytometer. The six channel images of cells from representative members of the double positive (DP), single positive (SP), and double negative (DN) populations identified using the ImageStream 100™ are shown in panels C, D and E, respectively.

Figures 4A – 4F show (A) a laser scatter analysis (forward scatter vs. side scatter) of the "DP gate" population of cells from Figure 3A by way of CellQuest™ software on data obtained from a FACSort™ cytometer; (B) a single color histogram of HLA class I-PE on DP cells from Figure 3A as measured using a FACSort™ cytometer; (C) backgating of HLA class I-PE$^+$ cells from Figure 4B onto the scatter histogram of Figure 4A, wherein HLA class I-PE$^+$ cells (*i.e.*, peroxide-treated, necrotic) are shown in red and HLA class I-PE$^-$ cells (*i.e.*, CPT-treated, apoptotic) are shown in blue; (D) a bivariate plot (scatter histogram) of the "Brightfield Area" vs. the "488 nm Scatter Peak Intensity" produced using IDEAS™ software on data obtained using the ImageStream™ cytometer; (E) a single color histogram of HLA class I-PE on DP cells from Figure 3D as measured using the ImageStream™ cytometer; (F) backgating of HLA class I-PE$^+$ cells from Figure 4E onto the scatter histogram of Figure 4D, wherein HLA class I-PE$^+$ cells (*i.e.*, peroxide-treated, necrotic) are shown in red and HLA class I-PE$^-$ cells (*i.e.*, CPT-treated, apoptotic) are shown in yellow.

Figure 5 shows resolution of live, early and late apoptotic, and necrotic cells using morphometric features based on scatter intensity, brightfield area, and nuclear area. Backgating of the four cell populations that had been identified using alternative criteria confirmed their identity as live cells and early apoptotic cells with the DN and SP cells,

respectively, shown in Figure 3B (shown in blue and green, center panel), and as necrotic and late apoptotic cells with cells contained in gates R3 and R4, respectively, shown in Figure 4 (shown in yellow and red, center panel).

DETAILED DESCRIPTION OF THE INVENTION

The instant disclosure relates to the use of both photometric and morphometric features derived from multi-mode imagery of objects (*e.g.*, cells) in flow to discriminate cell states or types, and cell features, in heterogeneous populations of cells, including both non-adherent and adherent cell types. A surprising result of the instant disclosure is the ability to discriminate between different cell states, such as differentiating and identifying live cells, necrotic cells, and cells in both the early and late stages of apoptosis, by using unique combinations of features provided in the ImageStream™ Multispectral Imaging Cytometer and the IDEAS™ data analysis software. Discussed in more detail below are single-step methods for basic and complex morphometric classification of objects in flow, which may be combined with comprehensive multispectral imagery and photometric features to allow, for example, the identification of different cell features and/or cell types or states not feasible with standard flow cytometry.

In the present description, any concentration range, percentage range, or integer range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer, etc.), unless otherwise indicated. As used herein, the term "about" means ± 15%. As used herein, the use of an indefinite article, such as "a" or "an", should be understood to refer to the singular and the plural of a noun or noun phrase (*i.e.*, meaning "one or more" of the enumerated elements or components). The use of the alternative (*e.g.*, "or") should be understood to mean either one, both or any combination thereof of the alternatives.

By way of background, methodologies for simultaneous high speed multispectral imaging in brightfield, darkfield, and four channels of fluorescence of cells in flow were recently developed (*see, e.g.*, U.S. Patent Nos. 6,211,955 and 6,249,341). Figure 1 illustrates an exemplary imaging system (*e.g.*, the ImageStream platform). Cells are

hydrodynamically focused into a core stream and orthogonally illuminated for both darkfield and fluorescence imaging. The cells are simultaneously trans-illuminated via a spectrally-limited source (*e.g.*, filtered white light or a light emitting diode) for brightfield imaging. Light is collected from the cells with an imaging objective lens and is projected on a charge-coupled detector (CCD). The optical system has a numeric aperture of 0.75 and the CCD pixel size in object space is 0.5 microns square, allowing high resolution imaging at event rates of approximately 100 cells per second. Each pixel is digitized with 10 bits of intensity resolution, providing a minimum dynamic range of three decades per pixel. In practice, the spread of signals over multiple pixels results in an effective dynamic range that typically exceeds four decades per image. Additionally, the sensitivity of the CCD can be independently controlled for each multispectral image, resulting in a total of approximately six decades of dynamic range across all the images associated with an object.

Prior to projection on the CCD, the light is passed through a spectral decomposition optical system that directs different spectral bands to different lateral positions across the detector (*see, e.g.*, U.S. Patent No. 6,249,341). With this technique, an image is optically decomposed into a set of 6 sub-images, each corresponding to a different color component and spatially isolated from the remaining sub-images. This process allows for identification and quantitation of signals within the cell by physically separating on the detector signals that may originate from overlapping regions of the cell. Spectral decomposition also allows multimode imaging: the simultaneous detection of brightfield, darkfield, and multiple colors of fluorescence. This is exemplified in Figure 1, which depicts a red brightfield illumination source and the associated transmitted light images in the red detector channel adjacent to fluorescent and scattered light images in the other spectral channels. The process of spectral decomposition occurs during the image formation process rather than via digital image processing of a conventional composite image.

The CCD may be operated using a technique called time-delay-integration (TDI), a specialized detector readout mode that preserves sensitivity and image quality even with fast relative movement between the detector and the objects being imaged. As with any CCD, image photons are converted to photocharges in an array of pixels. However, in TDI

operation the photocharges are continuously shifted from pixel to pixel down the detector, parallel to the axis of flow. If the photocharge shift rate is synchronized with the velocity of the flowing cell's image, the effect is similar to physically panning a camera: image streaking is avoided despite signal integration times that are orders of magnitude longer than in conventional flow cytometry. For example, an instrument may operate at a continuous data rate of approximately 30 megapixels per second and integrate signals from each object for 10 milliseconds, allowing the detection of even faint fluorescent probes within cell images that are acquired at high-speed. Careful attention to pump and fluidic system design to achieve highly laminar, non-pulsatile flow eliminates any cell rotation or lateral translation on the time scale of the imaging process (*see, e.g.*, U.S. Patent No. 6, 532,061).

A real-time algorithm analyzes every pixel read from the CCD to detect the presence of object images and calculate a number of basic morphometric and photometric features, which can be used as criteria for data storage. Data files encompassing 10,000-20,000 cells are typically about 100 MB in size and, therefore, can be stored and analyzed using standard personal computers. The TDI readout process operates continuously without any "dead time", which means every cell can be imaged and the coincidental imaging of two or more cells at a time, as depicted in Figure 1, presents no barrier to data acquisition.

Such an imaging system can be employed to determine morphological, photometric, and spectral characteristics of cells and other objects by measuring optical signals, including light scatter, reflection, absorption, fluorescence, phosphorescence, luminescence, etc. As used herein, morphological parameters may be basic (*e.g.*, nuclear shape) or may be complex (*e.g.*, identifying cytoplasm size as the difference between cell size and nuclear size). For example, morphological parameters may include nuclear area, perimeter, texture or spatial frequency content, centroid position, shape (*i.e.*, round, elliptical, barbell-shaped, etc.), volume, and ratios of any of these parameters. Morphological parameters may also include cytoplasm size, texture or spatial frequency content, volume and the like, of cells. As used herein, photometric measurements with the aforementioned imaging system can enable the determination of nuclear optical density, cytoplasm optical density, background optical density, and the ratios of any of these values. An object being imaged can

be stimulated into fluorescence or phosphorescence to emit light, or may be luminescent wherein light is produced without stimulation. In each case, the light from the object may be imaged on a TDI detector of the imaging system to determine the presence and amplitude of the emitted light, the number of discrete positions in a cell or other object from which the light signal(s) originate(s), the relative placement of the signal sources, and the color (wavelength or waveband) of the light emitted at each position in the object.

The present disclosure provides methods of using both photometric and morphometric features derived from multi-mode imagery of objects in flow. Such methods can be employed as a cell analyzer to determine one or more cell states or types, and cell features, in heterogeneous populations of cells when entrained in a fluid flowing through an imaging system. As used herein, cell states or types may include live cells, cells early or late in the process of dying (*e.g.*, apoptotic cells or necrotic cells), cells propagating (*e.g.*, cells in different phases of division), populations and subpopulations of cells (*e.g.*, leucocyte subpopulations in blood), etc., and combinations thereof. However, it should also be understood that these exemplary methods might be used for imaging and distinguishing other moving objects that have identifiable photometric and morphometric features. As used herein, gating refers to a subset of data relating to photometric or morphometric imaging. For example, a gate may be a numerical or graphical boundary of a subset of data that can be used to define the characteristics of particles to be further analyzed. Here, gates have been defined, for example, as a plot boundary that encompasses viable (normal) cells as double negatives (DN gate), or early apoptotic cells as single positives (SP gate), or late apoptotic and necrotic cells as double positives (DP gate). Further, backgating may be a subset of the subset data. For example, a forward scatter versus a side scatter plot in combination with a histogram from an additional marker (*e.g.*, HLA-class I-PE) may be used to backgate a subset (*e.g.*, late apoptotic cells) within the initial subset (*e.g.*, late apoptotic and necrotic cells).

In using an imaging system as described herein, it should be made clear that a separate light source is not required to produce an image of the object (cell), if the object is luminescent (*i.e.*, if the object produces light). However, many of the applications of an imaging system as described herein will require that one or more light sources be used to

provide light that is incident on the object being imaged. A person having ordinary skill in the art will know that the location of the light sources substantially affects the interaction of the incident light with the object and the kind of information that can be obtained from the images on a TDI detector.

In addition to imaging an object with the light that is incident on it, a light source can also be used to stimulate emission of light from the object. For example, a cell having been contacted with probe conjugated to a fluorochrome (*e.g.*, such as FITC, PE, APC, Cy5, or Cy5.5) will fluoresce when excited by light, producing a corresponding characteristic emission spectra from any excited fluorochrome probe that can be imaged on a TDI detector. Light sources may alternatively be used for causing the excitation of fluorochrome probes on an object, enabling a TDI detector to image fluorescent spots produced by the probes on the TDI detector at different locations as a result of the spectral dispersion of the light from the object that is provided by prism. The disposition of these fluorescent spots on the TDI detector surface will depend upon their emission spectra and their location in the object.

Each light source may produce light that can either be coherent, non-coherent, broadband or narrowband light, depending upon the application of the imaging system desired. Thus, a tungsten filament light source can be used for applications in which a narrowband light source is not required. For applications such as stimulating the emission of fluorescence from probes, narrowband laser light is preferred, since it also enables a spectrally decomposed, non-distorted image of the object to be produced from light scattered by the object. This scattered light image will be separately resolved from the fluorescent spots produced on a TDI detector, so long as the emission spectra of any of the spots are at different wavelengths than the wavelength of the laser light. The light source can be either of the continuous wave (CW) or pulsed type, preferably a pulsed laser. If a pulsed type illumination source is employed, the extended integration period associated with TDI detection can allow the integration of signal from multiple pulses. Furthermore, it is not necessary for the light to be pulsed in synchronization with the TDI detector.

In the embodiments of the present invention, it is to be understood that relative movement exists between the object being imaged and the imaging system. In most cases, it

will be more convenient to move the object than to move the imaging system. However, it is also contemplated that in some cases, the object may remain stationary and the imaging system move relative to it. As a further alternative, both the imaging system and the object may be in motion, which movement may be in different directions and/or at different rates.

In certain aspects, there is provided a method for identifying a specific cell, comprising directing incident light at a cell, using a detector to obtain a side scatter image, and using the spatial frequency content of the side scatter image to identify a specific cell. Within certain embodiments, the methods of the instant disclosure may be used to identify a specific cell subpopulation that is part of larger heterogeneous cell population. For example, the methods of this disclosure may be used to identify a normal cell, a cell undergoing apoptosis (including early and late stage apoptosis), and a cell undergoing necrosis. Alternatively, the methods of the instant disclosure may be used to identify cells at particular stages of replication (S phase, G phase, M phase, etc.). Thus, in a heterogeneous population of cells, the methods of the invention may be used to identify at least one apoptotic cell and at least one necrotic cell and at least one normal (viable) cell. In addition, early stage and late stage apoptotic cells may be indentified.

In another aspect, the instant disclosure provides a method for identifying a specific cell, comprising directing incident light at a cell, using a detector to obtain a brightfield image, and using the spatial frequency content of the brightfield image to identify a specific cell. In certain embodiments, the spatial frequency content analyzed is of the nucleus. Any of the aforementioned embodiments may be used within the context of this aspect of the invention.

In a further aspect, the instant disclosure provides a method for identifying a specific cell, comprising contacting a cell with a nuclear marker, directing incident light at the marked cell, using a detector to obtain an image of the cell, and using the nuclear marker image in combination with the spatial frequency content of the cell image to identify a specific cell. Again, any of the previous embodiments may be used within this method. In certain embodiments, only a single nuclear marker is used, such as 7-AAD.

In any of the aforementioned methods, multiple images may be collected simultaneously. Furthermore, in any of the aforementioned methods, there is relative motion between the cell and the detector. In addition, in any of the aforementioned methods, the detector is a time delay integration charge-coupled detector.

The instant disclosure also provides a kit for use in a multispectral imaging system to identify a specific cell type, comprising a single nuclear marker, wherein a cell contacted with the single marker for a time sufficient to allow identification of an apoptotic cell or a necrotic cell with the multispectral imaging system, as described herein.

All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet, are incorporated herein by reference, in their entirety. The invention having been described, the following examples are intended to illustrate, and not limit, the invention.

EXAMPLES

EXAMPLE 1

INDUCTION OF APOPTOSIS

Human acute T leukemic Jurkat cell line was obtained from ATCC (Rockville, MD; catalog number CRL-1990) and maintained in RPMI 1640 (Gibco, Grand Island, NY) containing 5% fetal bovine serum, 1 mM sodium pyruvate (Mediatech, Herndon, VA), 100 μM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (BioWhittaker, Walkersville, MD) in 5% CO_2 atmosphere at 37°C. The density of exponentially growing cells was less than $3x10^5$ cells per ml at the time of all treatments. To induce apoptosis, cells were treated for 18 hours with 1 μM camptothecin (CPT, Sigma), a DNA topoisomerase I inhibitor.

EXAMPLE 2

INDUCTION OF NECROSIS

Human acute T leukemic Jurkat cell line was obtained from ATCC (Rockville, MD; catalog number CRL-1990) and maintained in RPMI 1640 (Gibco, Grand Island, NY) containing 5% fetal bovine serum, 1 mM sodium pyruvate (Mediatech, Herndon, VA), 100 μM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (BioWhittaker, Walkersville, MD) in 5% CO_2 atmosphere at 37°C. The density of exponentially growing cells was less than $3x10^5$ cells per ml at the time of all treatments. To induce necrosis, cells were treated for 1 hour with 0.3% hydrogen peroxide (Sigma, St. Louis, MO).

EXAMPLE 3

STAINING TO IDENTIFY APOPTOTIC CELLS AND NECROTIC CELLS

Control (untreated) cell, CPT treated (apoptotic) cells, and peroxide treated (necrotic) cells were independently counted and washed once in phosphate buffered saline

(PBS, Fair Lawn, NJ). Each cell group was resuspended at 10^7 cells/ml in annexin V Binding Buffer (BD Pharmingen, San Diego, CA) containing Alexa Fluor 488 annexin V (Molecular Probes, Eugene, Oregon) and 10 μM 7-aminoactinomycin D (7-AAD, Molecular Probes) for 10 minutes at room temperature. Necrotic cells were additionally stained with phycoerythrin (PE)-labeled anti-HLA-A, B, C (clone G46-2.6, BD Pharmingen; anti-HLA class I). Each cell group was washed in annexin V Binding Buffer, fixed in 2% paraformaldehyde (Sigma), and analyzed as either single populations or as a mixture by flow cytometry and immunofluorescence microscopy.

EXAMPLE 4

CONVENTIONAL FLOW CYTOMETRY AND IMAGING FLOW CYTOMETRY

For flow cytometry, cell fluorescence data excited by a 488 nm laser were acquired using the FACSort™ cytometer (BD Immunocytometry Systems, San Jose, CA) and analyzed using CellQuest™ (BD Immunocytometry Systems). For imaging flow cytometry, fixed cells at $5x10^7$ cells per ml were run at 100 cells per second on an ImageStream100™ ("Beta" version), and the data analyzed using the ImageStream Data Analysis and Exploration Software™ (IDEAS™).

EXAMPLE 5

INSTRUMENTATION FOR MULTISPECTRAL IMAGING FLOW CYTOMETRY

Figure 1 provides an exemplary layout of the ImageStream™ platform. Cells are hydrodynamically focused into a core stream and orthogonally illuminated for both darkfield and fluorescence imaging. The cells are simultaneously trans-illuminated via a spectrally-limited source (*e.g.*, filtered white light or a light emitting diode) for brightfield imaging. Light is collected from the cells with an imaging objective lens and is projected on a charge-coupled detector (CCD). The optical system has a numeric aperture of 0.75 and the CCD pixel size in object space is 0.5 microns square, allowing high resolution imaging at event rates of approximately 100 cells per second. Each pixel is digitized with 10 bits of

intensity resolution, providing a minimum dynamic range of three decades per pixel. In practice, the spread of signals over multiple pixels results in an effective dynamic range that typically exceeds four decades per image. Additionally, the sensitivity of the CCD can be independently controlled for each multispectral image, resulting in a total of approximately six decades of dynamic range across all the images associated with an object.

Prior to projection on the CCD, the light is passed through a spectral decomposition optical system that directs different spectral bands to different lateral positions across the detector (*see, e.g.*, U.S. Patent No. 6,249,341). With this technique, an image is optically decomposed into a set of 6 sub-images, each corresponding to a different color component and spatially isolated from the remaining sub-images. This is exemplified in Figure 1, which depicts a red brightfield illumination source and the associated transmitted light images in the red detector channel adjacent to fluorescent and scattered light images in the other spectral channels. The process of spectral decomposition occurs during the image formation process rather than via digital image processing of a conventional composite image.

The CCD is operated using time-delay-integration (TDI), in which image photons converted to photocharges in an array of pixels are continuously shifted (at a rate synchronized with the velocity of the flowing cell's image) from pixel to pixel down the detector and parallel to the axis of flow to avoid image streaking. For example, the instrument can operate at a continuous data rate of approximately 30 megapixels per second and integrate signal from each object for 10 milliseconds, which allows the detection of even faint fluorescent probes within cell images that are acquired at high speed. Attention to pump and fluidic system design to achieve highly laminar, non-pulsatile flow can eliminate cell rotation or lateral translation on the time scale of the imaging process (*see, e.g.*, U.S. Patent No. 6, 532,061). Every pixel read from the CCD is analyzed by a real-time algorithm that detects the presence of object images and calculates a number of basic morphometric and photometric features, which can be used as criteria for data storage. Data files encompassing 10,000-20,000 cells can be about 100 MB in size, and are stored and analyzed using standard personal computers.

EXAMPLE 6

IMMUNOFLUORESCENCE MICROSCOPY

Fixed control and treated cells were placed on a conventional glass slide (Erie Scientific, Portsmouth, NH), mixed 1:1 with Antifade (Molecular Probes) and covered with a cover slip. The cells were visualized at 400X using an Eclipse E600 (Nikon, Melville, NY) fluorescence microscope equipped with filters appropriate for Alexa Fluor 488 (535/40 nm emission) and 7-AAD (630/60 nm emission).

EXAMPLE 7

CONVENTIONAL ANALYSIS OF CELLS INDUCED TO UNDERGO APOPTOSIS OR NECROSIS

Jurkat T cells were treated with peroxide (to induce necrosis), CPT (to induce apoptosis, which contained cells in both early and late stages of apoptosis), or were untreated (control). The three cell populations were then stained with Alexa Fluor 488 annexin V and 7-AAD and evaluated by brightfield, darkfield, and fluorescence microscopy, and by conventional flow cytometry (Figure 2). The vast majority (>98%) of the control cells were viable at the time of staining, and were annexin V^-, 7-AAD^- (double negative, DN; Figure 2A). CPT-treated (apoptotic) cells had two populations of cells, those that were annexin V^+ (single positive, SP, or early apoptotic cells), and those that were annexin V^+, 7-AAD^+ (double positive, DP, or late apoptotic cells) (Figure 2B). Similar to late apoptotic cells, peroxide-treated (necrotic cells) also stained positively with both annexin V and 7-AAD (Figure 2C). However, the condensed, fragmented nuclei of late apoptotic cells could be easily distinguished from the intact nuclei of necrotic cells by immunofluorescence microscopy. In addition, apoptotic cells exhibited greater darkfield intensity and texture as compared to necrotic cells (see B and C image panels on right, respectively).

EXAMPLE 8

ANALYSIS OF HETEROGENEOUS CELL POPULATION (NORMAL, APOPTOTIC AND NECROTIC)

A mixture of control, apoptotic, and necrotic Jurkat cells (individually prepared as described in Examples 1 and 2) were analyzed in parallel by conventional flow cytometry

and on an ImageStream 100™ (Beta system, multispectral imaging flow cytometer). In this experiment, all cells were stained with Alexa Fluor 488-conjugated annexin V and 7-AAD. Necrotic cells were also stained with PE-conjugated anti-HLA class I before mixing the cell populations to aid in distinguishing necrotic cells from late stage apoptotic cells, and to permit "backgating" when necessary. On the ImageStream™, each cell was simultaneously imaged in darkfield (488 nm laser side scatter), green fluorescence (500-550 nm, annexin V channel), orange fluorescence (550-600 nm, PE channel), red fluorescence (600-650 nm, 7-AAD channel), and brightfield (660-720 nm). Cells were grouped into live (DN), early apoptotic (SP), or double positive (DP, which would include late apoptotic and necrotic cells) populations based on the total intensities of annexin V and 7-AAD staining.

Similar bivariate dot-plots of annexin V and 7-AAD staining were obtained in analyses from both the conventional flow cytometer (*see* Figure 3A) and the multispectral imaging flow cytometer (*see* Figure 3B). However, a unique aspect of data collected on the ImageStream™ is that each data point can be "clicked on" to observe the cell imagery associated with each data point. Consequently, each population gate can be used to perform a "virtual cell sort" by displaying individual images of cells that fall within each gate – for example, representative images of cells contained in the DP, SP and DN gates can be "virtually sorted," as shown in Figures 3C, D and E, respectively, with each image row representing a different cell. Early apoptotic cells (cells in the SP gate) appear slightly shrunken, with more complex brightfield and darkfield morphologies, as compared to live cells in the DN gate. The double positive (DP) population contains cells with two distinct morphologies – one containing small, irregularly shaped cells with condensed, fragmented nuclei; and a second containing larger cells with large, unfragmented nuclei that stained uniformly with 7-AAD. The morphology of these two populations of cells is consistent with cells in the late stage of apoptosis and necrosis, respectively. Thus, in the absence of imagery provided by the multispectral imaging flow cytometer, data obtained from a conventional flow cytometer does not permit discrimination of similarly stained cells, such as late apoptotic cells from necrotic cells.

EXAMPLE 9

CONVENTIONAL METHODS TO DISTINGUISH LATE APOPTOTIC AND NECROTIC CELLS

As noted in Example 8, although advanced apoptotic and necrotic cells differ morphologically, they cannot be distinguished based solely on annexin V and 7-AAD fluorescence. Plotting the mixed late apoptotic and necrotic DP population on a forward scatter vs. side scatter (FSC vs. SSC) plot reveals two distinct populations of cells (*see* Figure 4A). Analysis of the DP population of cells obtained on the conventional flow cytometer for staining with PE (which was used to stain only the necrotic subpopulation of cells) permits separation of the necrotic and apoptotic subpopulations of cells (Figure 4B). Backgating the PE positive necrotic population in blue reveals that the low SSC population consists of necrotic cells (Figure 4C). However, without the aid of an extra marker (in this case, anti-HLA class I-PE) or imagery as described in Example 8, data obtained from a conventional flow cytometer does not permit discrimination of similarly stained cells, such as late apoptotic cells from necrotic cells.

EXAMPLE 10

MULTISPECTRAL IDENTIFICATION OF LATE APOPTOTIC AND NECROTIC CELLS

Analysis of the DP population with IDEAS™ software for size (Brightfield Area) and Scatter Peak Intensity also revealed two populations of cells (Figure 4D). The nuclei of cells that fell within the high brightfield area, low scatter peak intensity area (R3) were intact, uniformly stained with 7-AAD, and had a morphology consistent with necrotic cells. The nuclei of cells that fell within the low brightfield area, high scatter peak intensity area (R4) were condensed and fragmented, and had a morphology consistent with cells in the late stages of apoptosis. Backgating PE-positive cells (identified in the histogram shown in Figure 4E) in yellow verified that R3 gated cells were derived from the necrotic treatment group (Figure 4F). This conclusion is further supported by morphologic examination of cells in the image galleries of the R3 and R4 gated cells, and confirms that the low area/high texture cells were apoptotic (HLA-class I PE^- cells containing fragmented 7-AAD staining nuclei; lower right gallery) while high area/low texture cells were necrotic (HLA class I-PE^+ cells

containing uniform 7-AAD staining nuclei; upper right gallery). Thus, the data obtained from mulitspectral imaging is provided in form that allows one to distinguish similarly stained cells, such as late apoptotic cells from necrotic cells.

EXAMPLE 11

COMPLEX MORPHOLOGIC FEATURE IDENTITY USING MULTISPECTRAL IMAGING

Multispectral image data collection not only enables calculation of standard intensity-based parameters and statistics employed in conventional flow cytometry, but also permits quantitation of numerous other morphologic features (*e.g.*, cell area, perimeter, aspect ratio, texture, spot counts, cell centroid, gradient intensity, spatial frequency). Using this capability, it is possible to distinguish all four cell populations (*i.e.*, live, early apoptotic, late apoptotic and necrotic cells) in a single step using morphologic features derived from 7-AAD, brightfield and darkfield imagery (and in the absence of other staining procedures often used) to "identify" apoptotic cells.

By subtracting the 7-AAD image area (nuclear size) from the Brightfield area (cell size), a value is obtained that is an indication of cytoplasmic size. When this complex morphologic feature (herein referred to as "Brightfield – 7-AAD Area") was used in conjunction with a feature derived from darkfield imagery (*i.e.*, 488 nm spatial scatter frequency, which is an indication of internal cell complexity or cell granularity), four subpopulations of cells became evident (*see* Figure 5). The 488 nm spatial scatter frequency can be calculated by computing the standard deviation of the individual pixel intensities within the segmented dark field image mask.

Live cells (depicted in blue, Figure 5, center panel) excluded the cell-impermeant 7-AAD fluorescent DNA binding dye, which minimized the nuclear image area and resulted in cells with a large calculated cytoplasmic area.

Early apoptotic cells (depicted in green, Figure 5, center panel) are just as effective as live cells at excluding 7-AAD, but their total brightfield area is slightly smaller due to the early stages of cytoplasmic blebbing, thereby resulting in an intermediate value for the "Brightfield - 7AAD Area" parameter. Also associated with the early stages of apoptosis is

a significant increase in 488 nm scatter peak intensity, which clearly separates these cells from live cells on the vertical axis of the dot-plot.

Necrotic cells (depicted in yellow, Figure 5, center panel) and late apoptotic cells (depicted in red, Figure 5, center panel) both had compromised membrane integrity, which permits free entry of 7-AAD and thus strong nuclear images of relatively large area, shifting these populations to the left on the dot-plot. However, these two cell populations can be clearly separated based on the peak intensity measurements derived from their 488 nm scatter parameters. Necrotic cells produce darkfield images of relatively low complexity compared to the more complex and heterogeneous darkfield images of late apoptotic cells, thus clearly separating the two populations in the vertical axis.

Inspection of the associated image galleries associated with these four gated populations of cells confirmed the classification of each population (*see* Figure 5, upper and lower panels).

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

1. A method for identifying a specific cell, comprising directing incident light at a cell, using a detector to obtain a side scatter image, and using the spatial frequency content of the side scatter image to identify a specific cell.

2. The method of claim 1 wherein there is relative motion between the cell and the detector.

3. The method of claim 1 wherein a specific cell subpopulation is identified with a heterogeneous cell population.

4. The method of claim 1 wherein the specific cell identified is an apoptotic cell.

5. The method of claim 4 wherein the apoptotic cell is an early stage apoptotic cell or a late stage apoptotic cell.

6. The method of claim 1 wherein the specific cell identified is a necrotic cell.

7. The method of claim 1 wherein the specific cell identified is at least one of an apoptotic cell and a necrotic cell.

8. A method for identifying a specific cell, comprising directing incident light at a cell, using a detector to obtain a brightfield image, and using the spatial frequency content of the brightfield image to identify a specific cell.

9. The method of claim 8 wherein there is relative motion between the cell and the detector.

10. The method of claim 8 wherein a specific cell subpopulation is identified with a heterogeneous cell population.

11. The method of claim 8 wherein the specific cell identified is an apoptotic cell.

12. The method of claim 11 wherein the apoptotic cell is an early stage apoptotic cell or a late stage apoptotic cell.

13. The method of claim 8 wherein the specific cell identified is a necrotic cell.

14. The method of claim 8 wherein the specific cell identified is at least one of an apoptotic cell and a necrotic cell.

15. The method of claim 8 wherein the spatial frequency content is of the nucleus.

16. A method for identifying a specific cell, comprising contacting a cell with a nuclear marker, directing incident light at the marked cell, using a detector to obtain an image of the cell, and using the nuclear marker image in combination with the spatial frequency content of the cell image to identify a specific cell.

17. The method of claim 16 wherein there is relative motion between the cell and the detector.

18. The method of claim 16 wherein a specific cell subpopulation is identified with a heterogeneous cell population.

19. The method of claim 16 wherein the specific cell identified is an apoptotic cell.

20. The method of claim 19 wherein the apoptotic cell is an early stage apoptotic cell or a late stage apoptotic cell.

21. The method of claim 16 wherein the specific cell identified is a necrotic cell.

22. The method of claim 16 wherein the specific cell identified is at least one of an apoptotic cell and a necrotic cell.

23. The method of claim 16 wherein a single nuclear marker is used.

24. The method of claim 16 wherein the single nuclear marker is 7-aminoactinomycin D.

25. The method according to any one of claims 16-24 wherein the images are collected simultaneously.

26. The method according to any one of claims 1-24 wherein the detector is a time delay integration charge-coupled detector.

27. A kit for use in a multispectral imaging system to identify a specific cell type, comprising a single nuclear marker, wherein a cell contacted with the single marker for a

time sufficient to allow identification of an apoptotic cell or a necrotic cell with the multispectral imaging system.

28. The kit of claim 27 wherein the single nuclear marker is 7-aminoactinomycin D.

ABSTRACT OF THE DISCLOSURE

Provided are methods for determining and analyzing photometric and morphogenic features of small objects, such as cells to, for example, identify different cell states.

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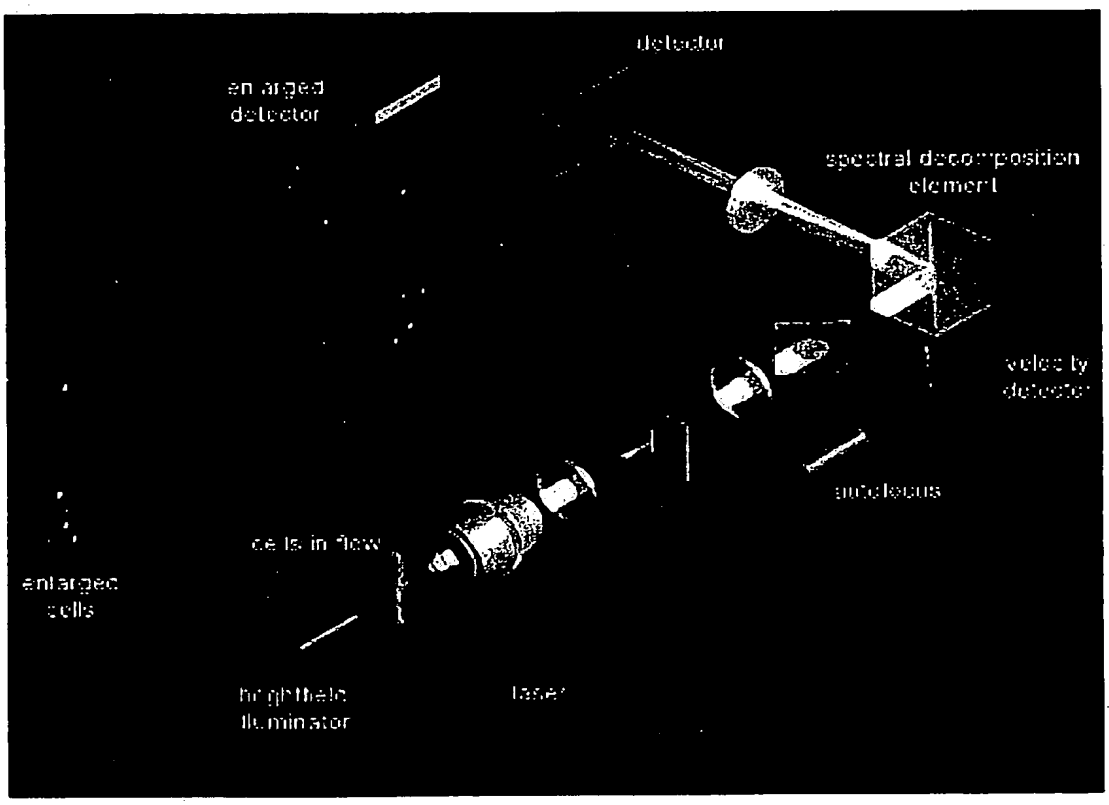
Figure 1.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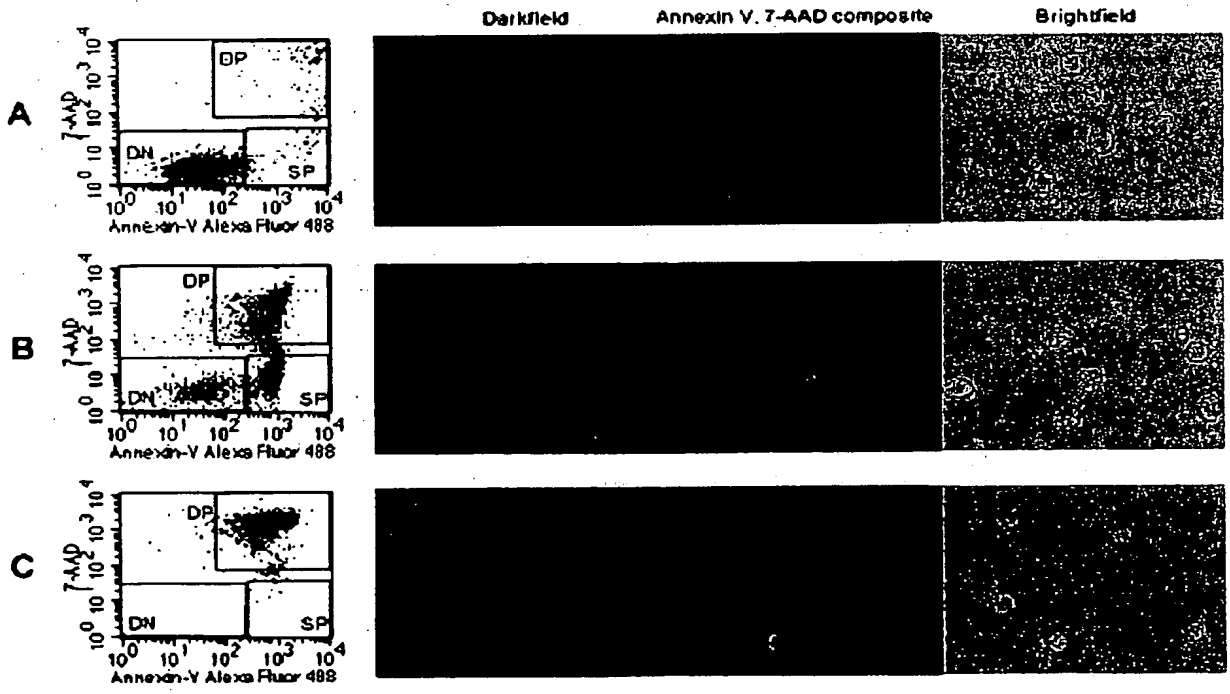
enlarged detector
spectral decomposition element
nucleus
cells in flow
enlarged cells
bright field illuminator
laser

Figure 2.


Darkfield
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Brightfield
A
B
C
DP
DN
SP
7-AAD
Annexin-V Alexa Fluor 488

Figure 3.

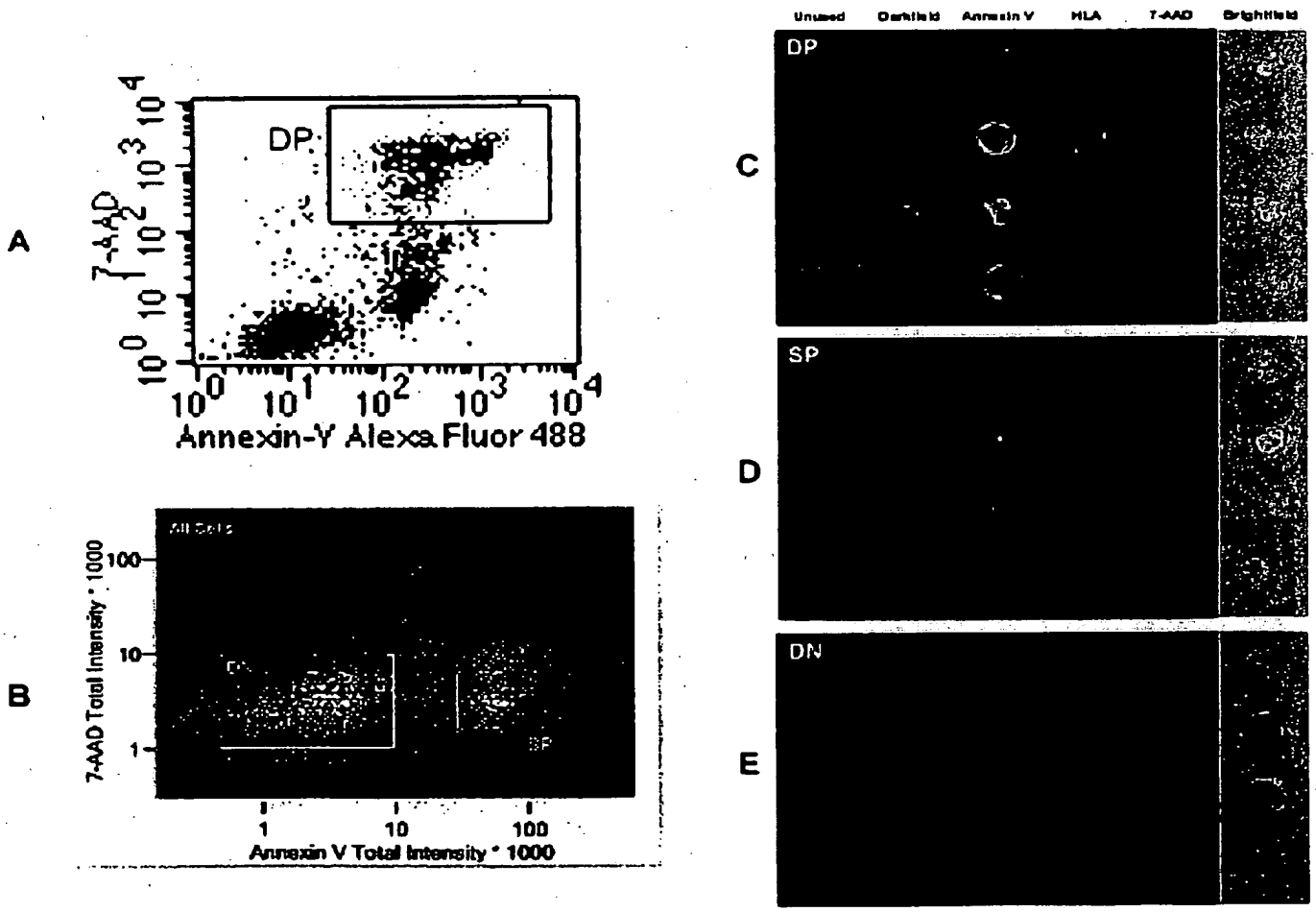
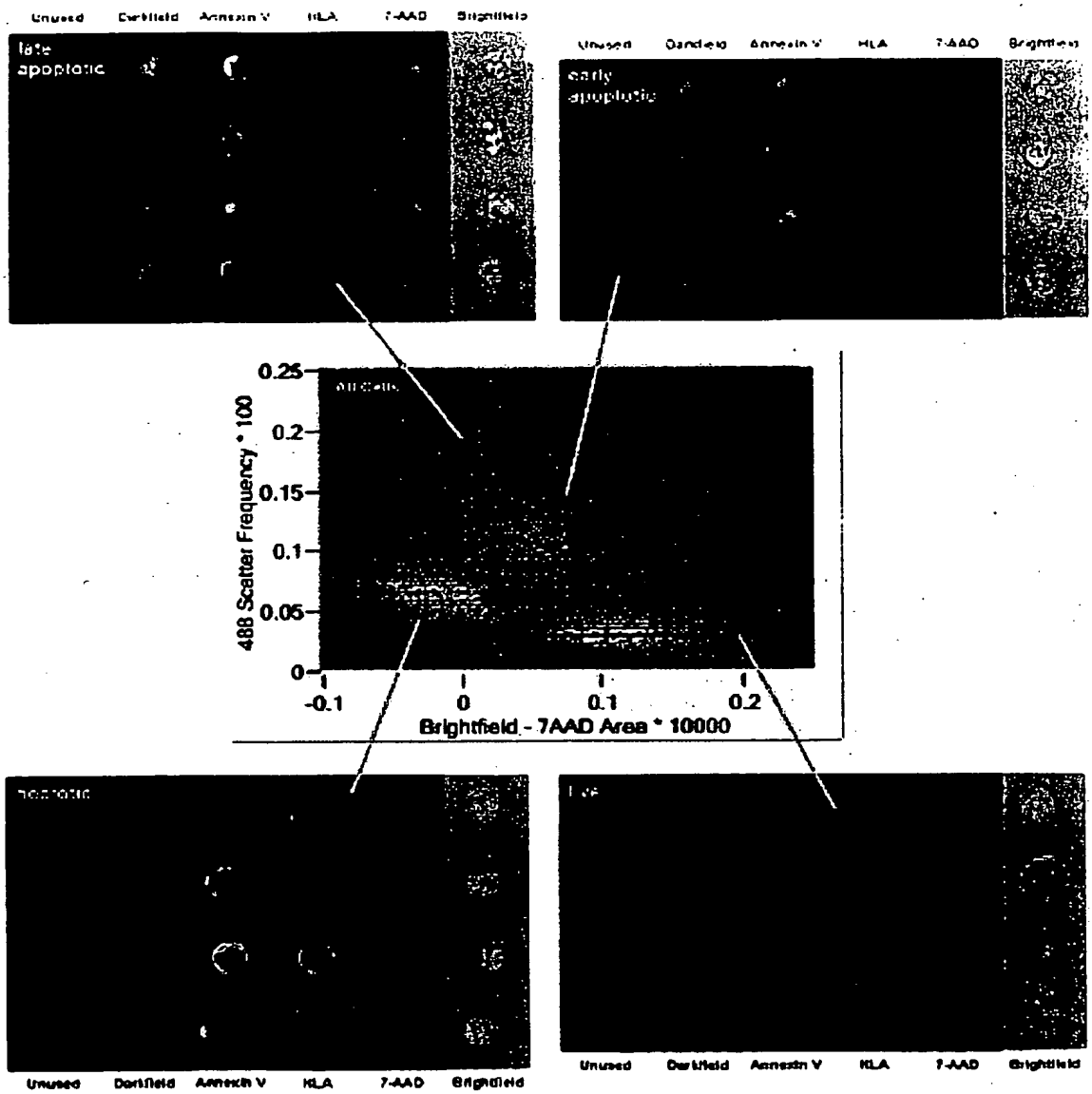

A
DP
7-AAD
Annexin-V Alexa Fluor 488
B
7-AAD Total Intensity * 1000
Annexin V Total Intensity * 1000
SP
C
DP
D
SP
E
DN

Figure 5.


Unused
Darkfield
Annexin V
HLA
7-AAD
Brightfield
late apoptotic
early apoptotic
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0.15
0.1
0.05
0
-0.1
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0.2
Brightfield - 7AAD Area * 10000
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Annexin V
HLA
7-AAD
Brightfield

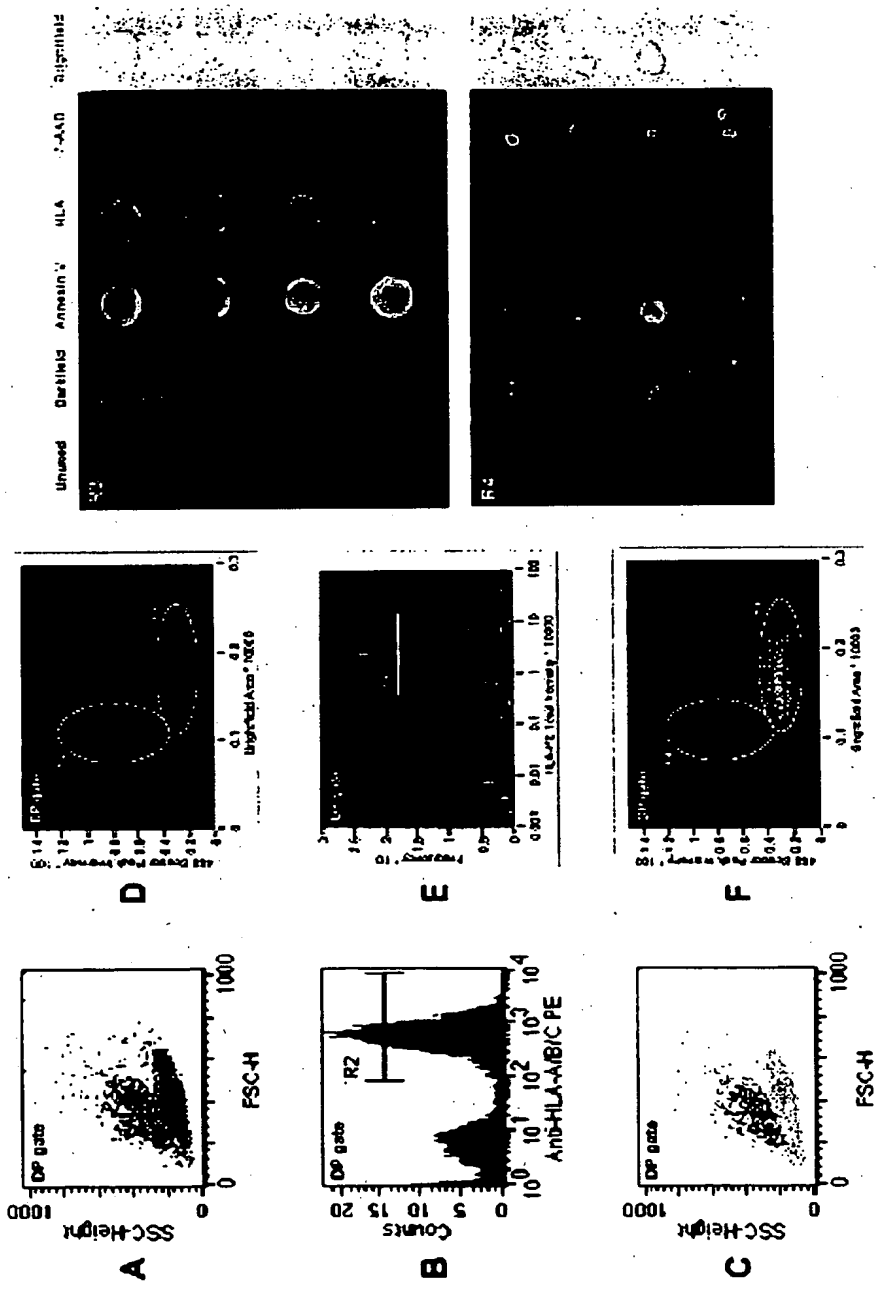

Figure 4.

APPLICATION DATA SHEET

Application Information

Application number::	
Filing Date::	
Application Type::	Provisional
Subject Matter::	Utility
Suggested classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R?::	None
Number of CD disks::	
Number of copies of CDs::	
Sequence submission?::	
Computer Readable Form (CRF)?::	No
Number of copies of CRF::	
Title ::	METHOD FOR IMAGING AND DIFFERENTIAL ANALYSIS OF CELLS
Attorney Docket Number::	120122.405P1
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Request for Non-Publication?::	No
Suggested Drawing Figure::	
Total Drawing Sheets::	5
Small Entity?::	Yes
Petition included?::	No
Petition Type::	
Licensed U.S. Gov't Agency::	
Contract or Grant No::	
Secrecy Order in Parent Appl.?::	No

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Country of mailing address::	US
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Correspondence Information

Correspondence Customer Number :: **00500**

Representative Information

Representative Customer Number::		**00500**

Domestic Priority Information

Application ::	Continuity Type::	Parent Application::	Parent Filing Date::

Foreign Priority Information

Country::	Application number::	Filing Date::	Priority Claimed::

Assignee Information

Assignee name::	Amnis Corporation
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State or Province of mailing address::	WA
Country of mailing address::	US
Postal or Zip Code of mailing address::	98121

105-93016

PATENT COOPERATION TREATY

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Date of mailing *(day/month/year)* 12 May 2005 (12.05.2005)	
Applicant's or agent's file reference 120122.405PC	**IMPORTANT NOTIFICATION**
International application No. PCT/US05/008870	International filing date *(day/month/year)* 16 March 2005 (16.03.2005)
International publication date *(day/month/year)*	Priority date *(day/month/year)* 16 March 2004 (16.03.2004)
Applicant AMNIS CORPORATION et al	

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Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
16 March 2004 (16.03.2004)	60/553,502	US	25 April 2005 (25.04.2005)

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